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Loop X/XI, the largest cytoplasmic loop in the membrane-bound melibiose carrier of *Escherichia coli*, is a functional re-entrant loop

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Abstract

The melibiose carrier of *Escherichia coli* is a membrane-bound sugar-cation cotransporter consisting of 12 transmembrane helices connected by cytoplasmic and periplasmic loops, with both N- and C-terminus on the cytoplasmic side. Using a functional cysteine-less carrier, cysteine was substituted individually for residues 347–378 that comprise the largest cytoplasmic loop X/XI. The majority of the cysteine mutants have good protein expression levels. The cysteine mutants were studied for their transport activities, and the inhibitory effects of two sulfhydryl reagents, PCMBS (7-Å long) and BM (29-Å long). Cysteine substitution resulted in substantial loss of transport in 12 mutants. While PCMBS caused significant inhibition in only two mutants, T373C and V376C, from the periplasmic side (in a substrate-protective manner), more extensive inhibition pattern was observed from the cytoplasmic side, in seven mutants: V353C, Y358C, V371C, Q372C, T373C, V376C and G378C, suggesting that these residues are along the sugar pathway in the aqueous channel, close to the cytoplasmic side. Furthermore, the inhibitory effect of BM on the inside-out vesicles of the above mutants was clearly less than that of PCMBS, suggesting channel space limitation to large molecules, consistent with those residues being inside the channel. Three second-site revertants (A350C/F268L, A350C/I22S, and A350C/I22N) were selected. They may suggest proximities between loop X/XI and helices I and VIII, in agreement with a re-entrant loop structure. Self thiol cross-linkings of the cysteine mutants on loop X/XI failed to form dimmers, suggesting that most of the loop is not surface-exposed from cytoplasmic side. Together, these results strongly indicated a functional re-entrant loop mechanistically important in Na^+-coupled transporters.

Keywords: Melibiose carrier; Cytoplasmic loop X/XI; Re-entrant loop; Membrane transport protein

1. Introduction

The melibiose carrier of *Escherichia coli* is a membrane-bound cation-substrate cotransport protein unique in its ability to utilize Na^+, Li^+ or H^+ as the coupling cation for the transport and accumulation of various α- and β-galactosides [1,2]. In the process, the cation travels down its electrochemical gradient into the cell through the transport protein, providing free energy to couple the entry of the sugar against its intracellular gradient. The accumulation of melibiose can reach 200-fold in Na^+-coupled cotransport. The Na^+ that enters the cell then exits via a Na^+/H^+ exchange protein in the membrane, thus maintaining its extracellular gradient. It is a secondary transporter that belongs to a family known as galactoside-pentoses-hexuronides (GPH) transport family, distinct from the predominant major facilitator superfamily (MFS) which includes the lactose permease of *E. coli* [2]. The transporter may serve as an evolutionary link between the proton-coupled transport that commonly occurs in primitive microorganisms and sodium-coupled transport prevalent in animal cells. The gene for the melibiose carrier (*melB*) was cloned and sequenced [3,4]. The primary sequence of the 473 residues in the protein indicates a high hydrophobicity with 70% nonpolar residues and a M.W. of 50 kDa. Hydropathy analysis combined with results from *melB–phoA* fusion

Abbreviations: PCMBS, *p*-chloromercuribenzene sulfonic acid; BM, (+)-biotinyl-3-maleimidopropionamidyl-3, 6-dioxaoctanediamine; MTSEA, (2-aminoethyl) methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl) methanethiosulfonate; MTSET, 2-(trimethylammonium) ethyl methanethiosulfonate

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[4–7] and protease cleavage experiments [8] predicted a secondary structure of 12 transmenbrane α-helices connected by hydrophilic loops, with both N- and C-terminus located on the cytoplasmic side of the membrane (Fig. 1). The carrier protein contains a single polypeptide [6]. FTIR analysis of the purified protein indicated up to 50% α-helical and 20% β-structures [9].

Na^+ and Li^+ increase the transport protein's affinity for melibiose, and the cations compete for the same binding site [10,11]. A 1:1 stoichiometry for Na^+/sugar cotransport was documented [11,12]. Investigations on cation recognition site through site-directed mutagenesis suggested that charged residues D19 (helix I), D55, D59 (both in helix II) and D124 (helix IV) form a network for cation binding/recognition [13–17]. Studies on the modification of charged residues in the transmembrane domains [18] and loop X/XI [46] showed that those charges cannot be replaced and suggested high degree of structural conservation on the native structures of these functional residues.

Cysteine-scanning has been performed on helices I [17], II [19], VI [20] and XI [21] in a functional cysteine-less carrier [22]. PCMBS inhibition of these cysteine mutants indicated that 30–35% of the residues in helices II and XI face the aqueous channel; helix I is entirely inside the aqueous channel, and helix VI is not in the aqueous channel. Second-site revertants (i.e., mutant with double mutations: the first mutation causes severe loss of the activity, but a second mutation at a different site restores partial or full activity) were selected from mutants with mutations in helices I [17] and XI [23]. Such double mutation sites in a restored active mutant may implicate proximity between the two helices in which the two mutations occur, presumably based on an interaction between the two mutations that can occur only when in proximity to each other. This interaction may result in a correct active conformation of the channel and hence the recovery of the activity (alternatively, the second-site mutation may cause a global conformational change that corrects the damage resulted from the first mutation). Chemical cross-linking confirmed proximity between helices I and XI [24] as was suggested by the second-site revertants. Using second-site revertants as a guidance for potential proximities between different helices, followed by confirmation with chemical cross-linkings, has been a valid strategy in our determination of the helix packing of melibiose carrier protein. Most recently, a projection structure at 8-Å resolution from 2-D crystals was obtained [25]. It revealed an asymmetric structure of 49 × 37 Å containing 12 helices that surrounded a curved central cavity presumably the aqueous channel. Significant extent of helix tilting may be implicated from the structure. The resolution was not enough to reveal any loop structures.

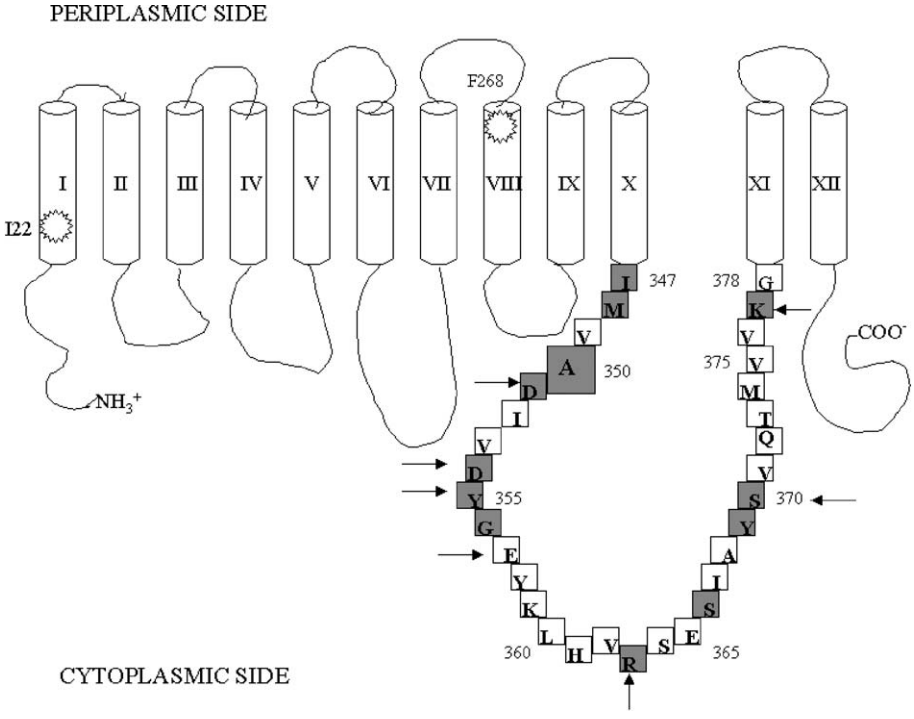

Fig. 1. Schematic representation of the *E. coli* melibiose carrier protein which has 12 transmembrane helices connected by periplasmic and cytoplasmic loops with both N- and C-terminus located on the cytoplasmic side. Cytoplasmic loop X/XI is shown for its sequence, with the shaded squares for those cysteine mutants having less than 25% of the transport activity of the cysteine-less parental strain; blank squares for the cysteine mutants having good transport activities (greater than 25% of the activity of the parental strain); arrows indicating cysteine mutants with low carrier protein expression levels (less than 30% of the parental strain level). Six out of the seven mutants with low carrier protein level also have low transport activities. Big square (350) is a cysteine mutant A350C with low activity from which second-site revertants were selected. I22 (helix I) and F268 (helix VIII) are the two second sites. Either or both may be close to A350 in 3-D structure of the carrier protein.

The present study was prompted by a previous investigation on sugar recognition mutants selected from a particular phenotype [26]. Most of the mutants have good transport for melibiose, but have lost the transport of lactose, and fail to recognize a competitive inhibitor thiomethyl-β-galactoside (TMG). Among those mutants, V349M, I 352V, A368V, Y369F, Q372L, T373A, M374I, M374V, V376A, V376G, G378S are particularly interesting in that they are on loop X/XI. This prompts the possibility that this loop forms part of the sugar recognition site and is along the sugar pathway. In this study, the entire loop was scanned by cysteine mutagenesis, one at a time, to determine those residues that are important for transport, and the inhibition pattern by sulfhydryl reagents from both periplasmic and cytoplasmic sides. Inhibition of sugar transport suggests that the target residue lies in the sugar pathway. The asymmetric inhibition pattern from the two sides of the membrane suggests that the part of the loop where the target residues reside is close to the cytoplasmic side of the membrane. Second-site revertants A350C/F268L, A350C/I22N and A350C/I22S, selected from A350 which has low activity, suggest proximities between the N-end of loop X/XI and cytoplasmic side of helix I, and/or the N-end of loop X/XI and periplasmic side of helix VIII. Such proximity could be readily explained by a re-entrant loop structure in which part of the loop is in close contact with helices that line the aqueous channel.

2. Materials and methods

2.1. Reagents

Melibiose (6-*O*-α-galactopyranosyl-D-glucopyranoside), PCMBS (*p*-chloromercuribenzene sulfonic acid), hydrogen peroxide and iodoacetic acid were from Sigma. BM [(+)-Biotinyl-3-maleimidopropionamidyl-3, 6-dioxaoctanediamine] was from Pierce. [^{3}H] melibiose was a generous gift from Dr. Gerard Leblanc. Bacteriological media were from Difco. [^{35}S] Protein A was from Amersham. MTSEA [(2-aminoethyl) methanethiosulfonate], MTSES [sodium (2-sulfonatoethyl) methanethiosulfonate] and MTSET [2-(trimethylammonium) ethyl methanethiosulfonate] were from Toronto Research Chemicals, Inc. All other chemicals were of reagent grade.

2.2. Bacterial strains and plasmids

E. coli DW1 (LacI$^+$Δ*ZY*Δ*melAB*) [27] was used as the host strain for plasmid. The cysteine-less *melB* gene [22] was placed in vector pKK223-3 (Pharmacia Biotech). This plasmid was used as the starting material for mutagenesis. The Quick Change Mutagenesis kit (Stratagene) was used to replace target residues with cysteine. Mutagenesis primers (40–45 mers) were synthesized at Biopolymers Laboratory at Harvard Medical School. All mutageneses were confirmed by sequencing of the *melB* gene at the Micro Core Facility at Harvard Medical School, using primers that anneal at approximately 500-bp intervals.

2.3. Quantitation of the melibiose carrier proteins

The expression levels of the carrier protein in each strain were determined as previously described by Lolkema et al. [28]. In essence, a known quantity of cells was lysed with NaOH/SDS and neutralized on a nitrocellulose filter. The filter was incubated with BSA to block nonspecific bindings, and then with a polyclonal antibody directed against the C-terminal 10 residues of the carrier protein [29]. [^{35}S] Protein A was used to label the bound antibody, and the amount of label was determined by liquid scintillation counting. For the correction of the nonspecific adsorption, the value for the strain DW1/pKK233-2 (without *melB*) was deducted as the background control in each determination. Values for each mutant are presented as a percentage of the parental cysteine-less carrier protein expression.

2.4. Assays for melibiose transport by intact cells

The plasmid-containing strains were grown in LB medium (100 μg/ml ampicillin) until they reached log phase of growth (reading around 200 on a Klett-Summerson photoelectric colorimeter). The cells were centrifuged and washed twice with 100 mM MOPS buffer (pH 7 adjusted with Tris base) containing 0.5 mM $MgSO_4$. The cells were then resuspended in the same buffer to a cell density corresponding to about 1 mg dry wt/ml. Cations were added 10 min before the addition of sugar to a final concentration of 10 mM. [^{3}H] Melibiose (2 μCi/ml) was added to an aliquot of the cell suspension to a final concentration of 0.1 mM. After incubation for 10 min at room temperature, a 200-μl sample was filtered through a 0.65-μm cellulose nitrate filter (Sartorius). The filters were immediately washed with 4-ml buffer containing appropriate cation and counted in the presence of Liquiscint (National Diagnostics). The effect of sulfhydryl reagent PCMBS on transport was measured by preincubating the cell suspension with 100 μM PCMBS for 10 min in the presence of 10 mM Na^+ at room temperature. The radioactive melibiose was then added and the transport measured as described above. In the charge modification experiments, 5 μl of MTSEA, MTSES, MTSET or iodoacetic acid (50 mM stock solution) was added to the cell suspension (containing 10 mM Na^+) to a final concentration of 1 mM, and incubated at room temperature for 30 min before adding melibiose. To the controls, 5 μl of water was added and the samples were handled under the same condition. For H_2O_2 oxidation experiment, a small volume of stock solution of 5 M H_2O_2 was added to the cell suspension (containing 10 mM Na^+) to a final concentra-

tion of 100 mM. After 5-min incubation at room temperature, the mixture was centrifuged at high speed for 1 min and the supernatant was removed. The pellets were washed with MOPS/Na^+ to remove the residual H_2O_2. The pellets were then resuspended in MOPS/Na^+ solution before the addition of melibiose. The volume of intracellular water, 0.48 μl/6×10^8 cells, was used for sugar accumulation calculation.

2.5. Assay for melibiose transport in inside-out membrane vesicles

Forty milliliters of plasmid−containing cells was grown in LB containing ampicillin to log phase, and harvested and washed with MOPS buffer containing 10 mM Na^+ and 0.5 mM $MgSO_4$, and resuspended in 10 ml of the above MOPS buffer containing 250 mM sucrose. Inside-out vesicles were prepared by passing the cell suspension through a French Press (SLM-Aminco) at 8000 psi [30] in a cold room. Unbroken cells were removed by centrifugation at 12 000 × *g* for 15 min at 4 °C. The vesicles were assayed for transport immediately afterwards. An aliquot of vesicles (310 μl) was warmed to room temperature for 10 min and the sulfhydryl reagents were added to a final concentration of 100 μM. The suspension was incubated for 10 min with PCMBS and 2 h with BM. [^{3}H] melibiose (40 μCi/ml) was then added to a final concentration of 50 μM. After 60 s, the vesicle suspension was filtered through a 0.22-μm nitrocellulose GSTF filter (Millipore), and the filter was washed with MOPS buffer containing sucrose. The filter was then counted in Liquiscint with 0.1% Triton X-100. The PCMBS and BM samples were prepared in distilled water. To the controls, the same small volume of water was added instead of the inhibitor.

2.6. Melibiose protection against PCMBS inhibition

A number of PCMBS concentrations were tested on both the intact cells and the inside-out vesicles of T373C and V376C. The lowest concentration that caused over 50% inhibition was used in the assay, using overlay method. To an aliquot of cell suspension (in the presence of 10 mM Na^+) previously warmed up to the room temperature, a small volume of melibiose was added to a final concentration of 1 mM. After 1 min, PCMBS was added and the suspension was let stand for 10 min. Then a 200-μl sample was loaded on the filter (the same filter used for the transport assay), filtered, and covered with 50-μl [^{3}H] melibiose (0.1 mM) for 30 s Then the liquid was filtered and the spot was washed with 4-ml MOPS (containing 0.1 mM Na^+). The filter was counted as described in the previous section. For the control samples, the same small volumes of water were added instead of the protection melibiose and PCMBS. The same procedure applies to the membrane vesicle assays, except that the protection melibiose concentrations were 1 and 3 mM, and that the incubation time with [^{3}H] melibiose on the 0.22-μm GSTF filter was 1 min.

2.7. Self thiol cross-linkings of the loop

Inside-out membrane vesicles (200 μl) were treated with 2-μl ethanolic iodine solution (5 mM) to a final concentration of I_2 of 50 μM. The controls were treated with 2-μl ethanol without I_2. If melibiose was applied (to a final concentration of 2 mM), the sample was let stand for 10 min at room temperature before adding the I_2. The samples were let stand at room temperature for 1 h for the cross-linking to complete and then loaded on a SDS-10% PAGE and run immediately in buffer without reducing reagent. Biotinylated M.W. markers (Cell Signaling Technology) were used to calibrate the size of the protein bands. The gel was then transferred onto a PVDF (Millipore) membrane for Western blot. Anti-MelB C-terminus antibody and anti-biotin antibody were used to label the carrier protein bands and the M.W. ladders, respectively. A second antibody, horseradish peroxidase-linked anti-rabbit IgG, was applied to visualize the protein bands through luminol/peroxide reaction (Cell Signaling Technology).

2.8. Selection for second-site revertants

Plasmids from inactive mutants were transformed into *E. coli* strain DW1/pSU*melA* (*melA* for α-galactosidase, which degrades melibiose to glucose and galactose; these sugars are metabolized to cause red coloring on MacConkey plate) and let grow at room temperature on MacConkey plate (Difco) containing 1% melibiose. The colonies were initially white. After 5–10 days, small brown colonies may appear and they were restreaked at least twice on fresh MacConkey plates to purify. Plasmids were extracted from single red colonies, and diluted 10,000 times, and then transformed back into DW1/pSU*melA* cells [44]. Plasmids from single red colonies were extracted and sequenced to identify second-site mutations.

3. Results

3.1. Protein expressions and transport activities of the cysteine mutants

The majority of the cysteine mutants have protein expression levels greater than 30% of the parental strain level (Table 1). Seven of the mutants (D351C, D354C, Y355C, E357C, R363C, S370C and K377C) have significantly reduced expressions (below 30% of the parental level), among which five have their charges removed. Five of these low expression mutants (D351C, D354C, Y355C, R363C, and K377C) also have near-zero transport (Table 2). This suggests the importance of D351, D354, Y355, R363 and K377 in the maintenance of the

Table 1
Phenotype and expression by single cysteine substitution of cysteine-less melibiose carrier in loop X/XI

Cell	Colony color on melibiose MacConkey plate[a]	Carrier protein expression (% of cysteine-less parent)[b]
Cysteine-less parent	Red	100
I347C	Red centered	70 ± 12
M348C	Red	73 ± 11
V349C	Red	44 ± 4
A350C	White	43 ± 9
D351C	White	29 ± 5
I352C	Red	100 ± 18
V353C	Red	76 ± 8
D354C	White	12 ± 2
Y355C	White	20 ± 4
G356C	White	37 ± 7
E357C	Red	25 ± 1
Y358C	Red	86 ± 4
K359C	Red	34 ± 8
L360C	Red	45 ± 9
H361C	Red	102 ± 11
V362C	Red	74 ± 11
R363C	White	24 ± 7
S364C	Red	53 ± 11
E365C	Very light pink	47 ± 9
S366C	Red centered	31 ± 4
I367C	Red	63 ± 11
A368C	Red	121 ± 3
Y369C	Red centered	50 ± 10
S370C	Red centered	23 ± 5
V371C	Red	99 ± 19
Q372C	Red	65 ± 10
T373C	Red	86 ± 13
M374C	Red centered	67 ± 17
V375C	Red centered	116 ± 12
V376C	Red	104 ± 4
K377C	White	7 ± 2
G378C	Red	139 ± 16

[a] Cell tested was DW1/pSUmelA with mutant plasmid. The MacConkey plate contains 1% melibiose.
[b] In DW1 cells, the standard deviation was calculated on the basis of three measurements.

protein activity and/or stability. Table 2 shows that altogether, 12 of the cysteine mutants located throughout the loop have each lost 80% or more of the Na^+-coupled transport (I347C, M348C, A350C, D351C, D354C, Y355C, G356C, R363C, S366C, Y369C, S370C and K377C; see Fig. 1) as compared with the parental strain. This may reflect the wide extent of the involvement of the loop in the maintenance of transport. In Q372C and V376C, Li^+ couples significantly higher accumulation of melibiose than Na^+ does, which is the reverse of the normal case. This may suggest that the two residues, 372Q and 376V, are either at or near the cation recognition site, or alternatively their changes influence the cation recognition site. From the periplasmic side, PCMBS inhibits only three mutants: Q372C is weakly inhibited, whereas T373C and V376C are significantly inhibited.

3.2. Inhibition from the cytoplasmic side by PCMBS and BM using inside-out membrane vesicles

The inhibitory effect of PCMBS (7 Å) on the transport was tested on 18 cysteine mutants having at least one third of the parental strain activity as intact cells (Table 3A). Experience from previous work tells that the transport

Table 2
Transport of melibiose[a] by cysteine mutants[b] in loop X/XI with three cations[c] and inhibition by PCMBS[d] (In/Out)[e]

Cell	H^+	Li^+	Na^+	PCMBS
Cysteine-less parent	8 ± 0.1	143 ± 2	157 ± 6	147 ± 0
I347C	1 ± 0.2	11 ± 0.1	14 ± 0.4	12 ± 0
M348C	2 ± 0	24 ± 0.3	28 ± 5	29 ± 0.5
V349C	23 ± 1	185 ± 4	203 ± 13	201
A350C	4 ± 0	35 ± 2	25 ± 1	22 ± 3
D351C	0	0	0.2	0
I352C	16 ± 1	221 ± 15	207 ± 9	207 ± 1
V353C	6 ± 0	83 ± 4	103 ± 5	94 ± 9
D354C	0	0.3	0.4 ± 0.1	0.3 ± 0.1
Y355C	0	0.3	0.8 ± 0.1	0.7 ± 0.1
G356C	0.7 ± 0.1	5 ± 0.4	11 ± 0.3	12 ± 0.1
E357C	3 ± 0.2	28 ± 1	52 ± 3	50 ± 9
Y358C	8 ± 0.1	54 ± 2	77 ± 2	73 ± 6
K359C	6 ± 1	52 ± 1	99 ± 4	96 ± 6
L360C	7 ± 1	101 ± 2	98 ± 11	93 ± 19
H361C	12 ± 2	157 ± 11	154 ± 12	146 ± 10
V362C	8 ± 0.6	90 ± 1	101 ± 4	100 ± 5
R363C	0	0.3 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
S364C	7 ± 0.1	101 ± 7	199 ± 23	185 ± 1
E365C	12 ± 1	51 ± 1	70 ± 1	67 ± 3
S366C	2 ± 0.3	25 ± 1	23 ± 0	21 ± 0
I367C	3 ± 0.4	40 ± 3	66 ± 1	65 ± 8
A368C	14 ± 1	192 ± 10	229 ± 12	232 ± 0
Y369C	4 ± 0.6	32 ± 0	31 ± 2	25 ± 0.6
S370C	0.2	18 ± 0	18 ± 0	17 ± 1
V371C	18 ± 0.2	118 ± 5	99 ± 2	99 ± 5
Q372C	26 ± 2	103 ± 15	65 ± 11	43 ± 2
T373C	7 ± 0.2	110 ± 7	135 ± 4	3 ± 0.3
M374C	15 ± 1	99 ± 0.4	114 ± 3	102 ± 7
V375C	4 ± 0	28 ± 4	67 ± 1	63 ± 1
V376C	22 ± 4	243 ± 13	173 ± 13	91 ± 3
K377C	0.3 ± 0.1	0.3 ± 0	1 ± 0	0
G378C	9 ± 1	139 ± 11	116 ± 2	118 ± 7

The standard deviation was calculated on the basis of two or three measurements.
[a] Melibiose concentration was 0.1 mM.
[b] Cells used were DW1 with mutant plasmids.
[c] Na^+ and Li^+ concentrations were 10 mM; H^+ was from 100 mM MOPS (pH 7).
[d] PCMBS concentration was 100 μM, in the presence of 10 mM Na^+.
[e] The ratio of melibiose concentrations inside over outside the cell.

Table 3A
PCMBS[a] (7-Å long) inhibition of inside-out membrane vesicles

Cell	%Transport activity	
	Without PCMBS	With PCMBS
melB (c-less parent)	100	101
V349C*	132 ± 6	98 ± 8
I352C*	182 ± 5	146 ± 1
V353C**	51 ± 14	25 ± 6
Y358C**	67 ± 4	45 ± 2
K359C	36 ± 4	40 ± 8
L360C	46 ± 3	37
H361C	82 ± 10	86 ± 8
V362C	50	47 ± 10
S364C	62	50 ± 7
I367C*	40 ± 3	30 ± 1
A368C	124 ± 6	111 ± 11
V371C**	52 ± 6	30 ± 0.1
Q372C**	29 ± 1	7 ± 3
T373C***	107 ± 22	10 ± 5
M374C*	47 ± 1	34 ± 6
V375C	5	8 ± 2
V376C***	72 ± 0.1	10 ± 1
G378C**	80 ± 2	46 ± 4

(*) Weak inhibition; (**) moderate inhibition; (***) strong inhibition.
Cells tested were DW1 cells.
[a] PCMBS concentration was 100 μM.

activities of inside-out membrane vesicles made from cells with low activities are accordingly low. Since decent activity level is required to test the inhibition, those strains with low activities were not used in the assay. Eleven mutants were inhibited by PCMBS. V349C, I352C, I367C and M374C were weakly inhibited; Y353C, Y358C, V371C and G378C were moderately inhibited; Q372, T373 and V376 were strongly inhibited. PCMBS had no effect on the parental cysteine-less strain.

Eight of the mutants that were inhibited by PCMBS as inside-out membrane vesicles were further tested for inhibition by a much larger reagent BM (29 Å) (This BM is not the same as biotin maleimide from Sigma). This BM is not membrane-permeable [48]. Table 3B shows that the mutants all have reduced inhibition levels compared with those by PCMBS. Notably, in T373C and V376C, the inhibition levels by BM are reduced to moderate, as compared with the strong inhibition by PCMBS (Table 3A). For reasons unclear, BM also inhibits the cysteine-less parental strain to a small extent. The weak inhibitions of the cysteine mutants by BM may just be background inhibitions. Since T373C and V376C are significantly inhibited by both PCMBS and BM from the cytoplasmic side, and they are also inhibited strongly by PCMBS from the periplasmic side (Table 1), the two residues, 373T and 376V, are most likely accessible from both sides of the channel. Many of the cysteine mutants were not inhibited by PCMBS or BM, therefore it is not known whether those residues are either reactive to or accessible to thiol reagents. But for the cysteine mutants that are inhibited, those residues are most likely accessible and reactive to the reagents.

Table 3B
Inhibition of inside-out vesicles by BM[a] (29-Å long)

Cell	%Transport activity	
	without BM	with BM
melB (c-less parent)	100	87 ± 11
I352C*	158 ± 9	138 ± 10
Y353*	33 ± 3	24 ± 3
Y358C*	32 ± 5	22 ± 2
A368C	93 ± 3	97 ± 13
V371C*	50 ± 4	39 ± 1
T373C**	104 ± 6	60 ± 16
V376C**	49 ± 8	13 ± 4
G378C*	81 ± 11	66 ± 3

(*) Weak inhibition; (**) moderate inhibition.
Cells tested were DW1 cells.
[a] BM concentration was 100 μM.

3.3. Melibiose protection against PCMBS inhibition

As shown in Table 4, melibiose protected against PCMBS inhibition of T373C and V376C from the periplasmic side. This suggests that the two residues, T373 and V376, are at or near the binding site of the substrate. However, from the cytoplasmic side (in inside-out vesicle assays), melibiose failed to protect the two sites, nor did it protect V353 (data not shown).

3.4. Second-site revertants

Cysteine mutants with low or near-zero melibiose accumulation, as indicated by white colonies on MacConkey plate (Table 1) and by transport activity assay (Table 2), were used to screen second-site revertants from. Among all the strains screened, only A350C yielded three second-site: A350C/F268L, A350C/I22N and A350C/I22S (Table 6). These revertants clearly have recovered at least part of the activity on MacConkey plate (containing 29 mM melibiose and more than 86 mM Na^+). The second sites are located in helix VIII at the periplasmic side (F268) and helix I near the cytoplasmic side (I22). This may suggest that 350A is close to the cytoplasmic side of helix I and/or periplasmic side of helix VIII. In future

Table 4
Melibiose protection against PCMBS inhibition of intact cells

Cell	%Remaining transport activity	
	PCMBS	Melibiose + PCMBS
T373C[a]	16 ± 2	34 ± 1
V376C[b]	27 ± 18	57 ± 19

Cells tested were DW1 cells. The standard deviation was calculated on the basis of five to six determinations. Although not shown here, in the inside-out membrane vesicle assays, melibiose did not protect against PCMBS inhibitions.
[a] PCMBS = 10 μM; melibiose = 1 mM.
[b] PCMBS = 100 μM; melibiose = 1 mM.

work, cross-linking experiments will be planned to test the proximities in question.

3.5. Modification of charged residues (using intact cells)

The loss of the negative charge due to cysteine substitution can be replaced by reaction between cysteine and MTSES or iodoacetic acid, or by oxidation of cysteine with hydrogen peroxide to sulfinic and/or sulfonic acids. The restoration of the original charges can help recover transport activity in some cases in membrane transport proteins, as demonstrated in lactose permease [31,32]. Table 5A shows that none of these reagents recovered any transport activity of D351C and D354C. Positive charge loss due to cysteine substitution can be rescued by reaction between cysteine and MTSEA or MTSET. Table 5B shows that neither reagent restored the transport of R363C. K377C has very low protein expression (7% of normal, see Table 1) and was not tested. All the five modifying reagents were found to be able to enter deep into the aqueous channel, as indicated by their strong inhibitory effects on I22C, a mutant (with cysteine mutation in helix I near the cytoplasmic side, see Ref. [17]) with near parental strain transport activity and 100% inhibition by PCMBS (unpublished observations; also see Ref. [18]). Although not shown here, none of the above modifying reagents recovered any transport activities on the inside-out membrane vesicles of those mutants in Tables 5A and 5B [46]. Although these thiol reagents can reach deep down into the aqueous channel as suggested by I22C results, it is, however, not impossible that the cysteine residues on the loop are not reactive to the reagents, or are in contact with other residues in the channel and therefore not accessible to the reagents.

3.6. Self thiol cross-linkings

Membrane vesicles of 25 cysteine mutants on loop X/XI (V349C, A350C, D351C, I352C, V353C, D354C, Y355C, G356C, Y358C, K359C, L360C, H361C, V362C, R363C, S364, S366C, I367C, A368C, S370C, V371C, Q372C, T373C, M374C, V375C and V376C) were tested for dimer formation by thiol cross-linking catalyzed by iodine, in the absence of melibiose. Among them, nine cysteine mutants which have very good melibiose transport activities (V353C, K359C, L360C, H361C, V362C, S364C, A368C, V371C and T373C) were tested for I_2-catalyzed thiol cross-linking in the presence of melibiose. On the SDS 10% PAGE, the monomer of the melibiose carrier protein has an apparent M.W. of 35 kDa (consistent with the 32 kDa M.W. of lactose permease on the SDS 10% PAGE, see Ref. [33]). Fig. 2A shows that in the absence of the substrate, no dimer formation was detected in all but two of the tested cysteine mutants. K359C showed a clear band at 75 kDa, in the absence and presence of iodine. The 75-kDa band was proven to be a dimer formed by joining two monomers through a disulfide bond (if 40 mM dithiothreitol (DTT) was added to the sample 15 min prior to the electrophoresis, the 75-kDa band completely disappeared as a result; unpublished observation). H361C showed a light band of dimer, both in the absence and presence of iodine. In the absence of iodine, the thiol cross-linking occurred by spontaneous oxidation. The cysteine-less carrier (MelB c-less) was used as a negative control. In the presence of substrate melibiose (2 mM), no dimer formation was detected in the nine cysteine mutants (including K359C and H361C) that had good transport activities (melibiose evidently bound to these functional carriers), as shown in Fig. 2B.

Table 5A
Modification of negatively charged residues D351 and D354 on loop X/XI

Cell	Melibiose accumulation (in/out)			
	control[a]	MTSES[b]	ICH_2COOH[c]	H_2O_2[d]
melB (c-less)	183 ± 11	187	112	176 ± 4
D351C	0.2	0	0.6	0.1
D354C	1.8	1.6	0.8	1.7

Cells tested were DW1 cells.

[a] No modifying reagent added. All the tests used final concentrations of melibiose at 0.1mM and Na^+ at 10 mM.

[b] Final concentration was 1 mM. Incubation with the reagents was at room temperature for 30 min.

[c] Final concentration was 1 mM. Incubation with the reagents was at room temperature for 30 min.

[d] Final concentration was 100 mM. Incubation with H_2O_2 was at room temperature for 5 min.

Table 5B
Modification of positively charged residue R368 on loop X/XI

Cell	Melibiose accumulation (in/out)		
	control[a]	MTSEA[b]	MTSET[c]
melB (c-less)	170	6	154
R363C	1.2	1.2	0.6

Cells tested were DW1cells.

[a] No modifying reagent added.

[b] Final concentration was 1 mM. Incubation with the reagents was at room temperature for 30 min.

[c] Final concentration was 1 mM. Incubation with the reagents was at room temperature for 30 min.

4. Discussion

Previous investigations on sugar recognition mutants of the melibiose carrier [26] found that mutations of eight residues on loop X/XI, which may or may not significantly change melibiose accumulation, caused a loss of the recognition of a competitive inhibitor TMG and the transport of lactose: V349M, I352V, A368V, Y369F, Q372L, T373A, M374I, M374V, V376G and V376A. This suggested an important functional role of the loop in transport. These residues either lie on the sugar pathway (the aqueous channel) being at or near the sugar recognition site (which requires that loop X/XI be a re-entrant loop), or, alternatively, their structural changes cause a conformational

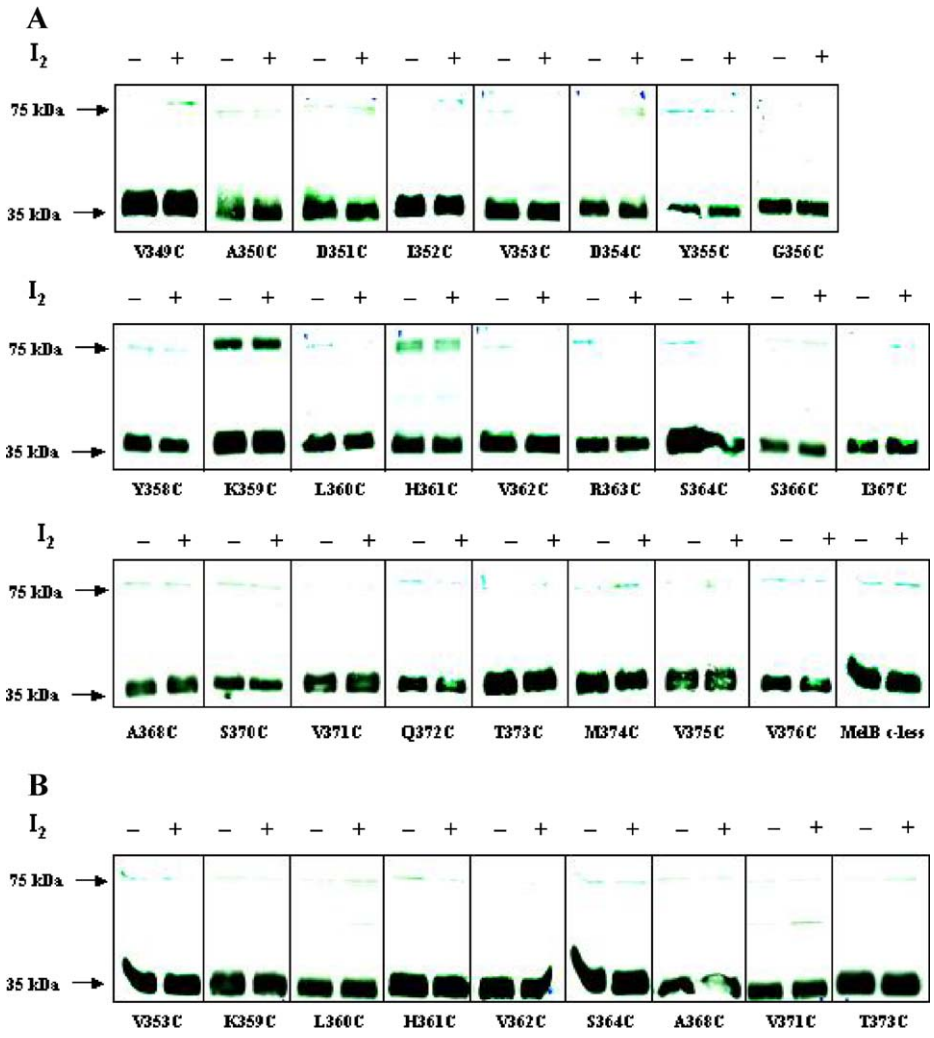

Fig. 2. Immunoblots of the attempted self thiol cross-linking of cysteine mutants on loop X/XI in the absence of substrate melibiose (A) and in the presence of 2 mM melibiose (B). Inside-out membrane vesicles were used. The nine cysteine mutants in (B) all have good transport activities, therefore melibiose binding should occur. As shown in (A), only 2 out of 25 cysteine mutants showed dimer formation: K359C had a moderate amount of dimer formation, and H361C had a small amount of dimer formation. The melibiose carrier protein has an apparent M.W. of about 35 kDa on SDS 10% PAGE. The dimer formed by cross-linking has a M.W. of 75 kDa (see Results for explanation).

change at the sugar recognition site at a different location; hence, a systematic investigation on the entire loop carried out by cysteine scanning. The protein expression levels of the cysteine mutants were generally good, with the majority over 30% of the parental strain level (Table 1). The cysteine substitution of four charged residues, D351, D354, R363 and E365, caused significant loss of melibiose transport. Eleven neutral residue substitution by cysteine also caused significant loss of melibiose transport: I347, M438, A350, Y355, G356, S366, I367, Y369, S370, Q372 and V375 (Tables 1 and 2). These results clearly indicate that loop X/XI is a functional loop.

Inhibition studies on the cysteine mutants were carried out using two membrane-impermeable sulfhydryl reagents PCMBS and BM. PCMBS inhibitions showed that from the periplasmic side, only three mutants were affected: Q372C had weak inhibition, T373C and V376C had significant inhibition (Table 2). The results are very consistent with those of MTSES and MTSET [46]. PCMBS is a relatively small molecule (7 Å) that can reach deep down into the aqueous channel, as was suggested by the finding that it interacts to a significant extent with F20C, A21C and I22C (which are near the cytoplasmic side) [17]. The finding that thiol reagents of varying sizes target the same residues suggests that Q372, T373 and V376 on the loop are quite accessible to the aqueous environment. Such small number of inhibition sites on the loop suggests that the majority of the residues on the loop are either in close contact with the lipid membrane and/or helices that face the aqueous channel, and therefore not readily available to react with PCMBS. Alternatively, the loop may be very close to the cytoplasmic side of the channel where PCMBS cannot easily have access to when it comes from the periplasmic side, even though it can reach near the cytoplasmic side.

However, for inside-out membrane vesicles, PCMBS inhibition occurred at more sites (Table 3A): Y353C, V371C, Q372C, T373C, V376C and G378C had significant inhibition; in addition, V349C, I352C, Y358C and M374C had weak to moderate inhibition. This is consistent with the assumption that the loop is close to the cytoplasmic side of the aqueous channel. Furthermore, the use of a large thiol-specific compound BM (29 Å) on the inside-out membrane vesicles of I352C, Y353C, Y358C, V371C, T373C, V376C and G378C (Table 3B) showed somewhat decreased inhibition, as compared with that by PCMBS for the same mutants. Particularly, two mutants, T373C and G378C, which were strongly inhibited by PCMBS by 90% and 42%, respectively, were now inhibited by BM by only 42% and 19%, respectively. Even though the precise size the aqueous channel is not known, the overall size of the channel section that comprises 12 helices is estimated to be 49×37 Å [25]. The aqueous channel must be much smaller in size. Therefore, the 29-Å BM does not seem to be able to access the aqueous channel as easily as the much smaller 7-Å PCMBS does. The decreased inhibition due to the increase in size of the inhibitor may reflect size limitation of the aqueous channel and is consistent with a re-entrant loop structure.

Intermolecular thiol cross-linking of the loops and the loop–helix junctures of the transmembrane helices provides critical evidence of whether those parts of the membrane protein are surface-exposed. This was demonstrated in lactose permease [33,34]. I_2-catalyzed thiol cross-linkings of 25 cysteine mutants on loop X/XI were performed. Twenty-three failed to produce dimers (Fig. 2A). A moderated amount of dimer was detected in K359C, and a small amount of dimer was found in H361C. Residues 359K and 361H are near the middle of loop X/XI (Fig. 3A). The

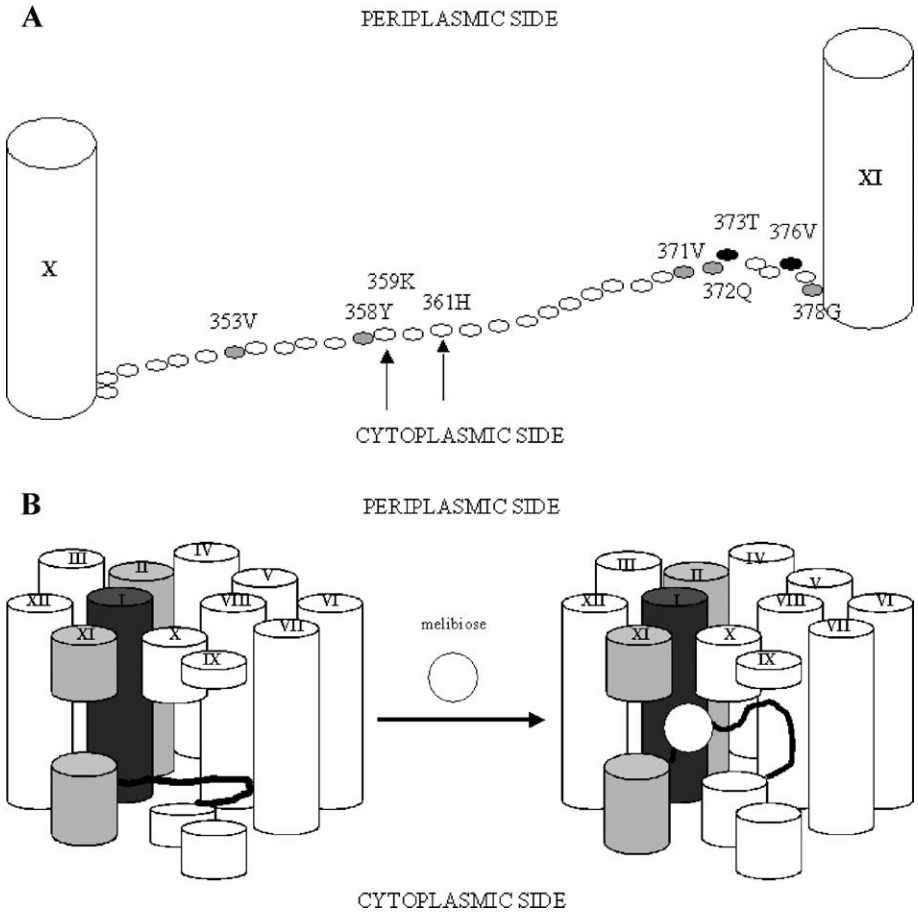

Fig. 3. A model for the melibiose carrier showing the re-entrant loop X/XI oriented inside the aqueous channel. (A) Loop X/XI is close to the cytoplasmic side of the membrane. Black residues 373T and 376V are accessible PCMBS from both sides of the membrane; gray residues 353V, 358Y, 371V, 372Q and 378G are accessible to PCMBS only from the cytoplasmic side. 359K and 361H indicated by arrows are surface-exposed (subject to thiol cross-linking) in the absence of melibiose. (B) A model for the melibiose carrier protein showing the relative arrangement of the 12 transmembrane helices and the cytoplasmic re-entrant loop X/XI (thick black curved line) in the aqueous channel (part of which is shaded) as well as a conformational change of the loop upon the binding of the melibiose which prevented previously surface-exposed residues from cross-linking. The model is corroborated by a number of previous findings including: helix I (dark gray) is entirely in the aqueous channel [17]; helices II and XI (gray) are partially in the aqueous channel [19,21]; helix VI is out of the aqueous channel [20]; helix I is close to helix XI (by cross-linking) [24]. The model also shows that six helices are on each side of a central cavity, consistent with a recent projection structure revealed by electron cryomicroscopy [25]. For the sake of simplicity, all other loops and the C- and N-terminus are omitted in the scheme.

addition of substrate melibiose (2 mM) to nine functionally very active membrane vesicles (including K359C and H361C) with the intention to bring about some conformational change of the loop relinquished dimer formations in K359C and H361C (Fig. 2B). These results strongly suggest that most of loop is not surface-exposed, unavailable for intermolecular contact, consistent with re-entrant loop structure. In addition, the binding of melibiose to the carrier caused some conformational changes to the loop such that the two surface-exposed residues 359K and 361H (when in the absence of melibiose) were tucked away from the surface, as illustrated in Fig. 3B. The conformational change of the loop (upon the binding of substrate) as deduced from the change of cross-linking pattern reflects the dynamic aspect of the loop.

Efforts were made on the screening of second-site revertants from inactive cysteine mutants on MacConkey plates. Three second-site revertants (as red colonies) were obtained from A350C (white colonies): A359C/F268L, A350C/I22N and A350C/I22S (Table 6). The position 350 is near the N-end of loop X/XI, three residues away from the juncture of helix X. One second-site, 22, is in helix I, near its cytoplasmic side; the other second-site, 268, is in helix VIII at its periplasmic side (Fig. 1). Second-site revertants may reflect proximity between the mutation sites, or helices in which the two mutations occur, as demonstrated by cross-linking between helices I and XI [24] as an example. The suggested proximities between A350 and the cytoplasmic side of helix I, and/or A350 and the periplasmic side of helix VIII, would require that the N-end of loop X/XI be inside the aqueous channel.

The attempt to restore charges to residues through cysteine modification failed to recover transport activities of inactive mutants D351C, D354C and R363C (Tables 5A and 5B). A previous study on the modification of nine charged residues in the transmembrane domains [18] indicated that both the charge and the side chain structure of those charged residues are important for the transport function, distinct from lactose permease which tolerates several charge modifications [31,32]. The same conclusion could be reached on the charged residues on loop X/XI. However, it is also possible that those cysteine residues that replaced the charged residues are in close contact with the lipid membrane or helices that comprise the inside wall of the aqueous channel, and therefore not reactive to modifying reagents.

Table 6
Phenotype and carrier expression of second-site revertants of A350C

Cell	Color of colony on MacConkey plate	Helix position of second-site	%Expression level
melB (c-less)	Red		100
A350C	White		43 ± 9
A350C/F268L	Red	VIII	176 ± 8
A350C/I22N	Red	I	108 ± 2
A350C/I22S	Red	I	144 ± 15

Cells tested were DW1/pSUmelA with mutant plasmids.

Functional re-entrant loop structures have been speculated and postulated for both intracellular and extracellular loops in a number of membrane proteins, including LacS (loop X/XI) [2, 45], type IIa Na^+/Pi cotrnsporter [35,36], glutamate transporters [37–39] and human sodium/proton exchanger isoform 1[40]. In aquaporin-1 [41] and potassium channel [42], re-entrant loops were revealed by crystallography. A common feature of the postulated re-entrant loops in transporters is that the in-pore loop structures from the two opposite sides of the aqueous channel contain functionally important residues accessible to reagents from both sides of the membrane, forming part of the substrate pathway. Such structure may form the basis for the postulated mechanism of sodium-coupled transport whereby an alternating exposure of the substrate binding site to the periplasmic and cytoplasmic sides of the membrane drives the transport cycle. T373C and V376C on loop X/XI in this study are of particular interest in this context. Their accessibility from both sides of the membrane to water-soluble PCMBS (similar in size with melibiose) may suggest that T373 and V376 are at a special location in the substrate pathway of the melibiose carrier. This is also corroborated by the finding that Li^+-coupled melibiose accumulation is significantly higher than Na^+-coupled accumulation in Q372C and V376C (Table 2), suggesting that the two residues may be involved in cation recognition.

Moreover, T373C and V376C are protected by substrate against PCMBS inhibition from the periplasmic side of the protein, consistent with their positions being at or near the melibiose binding site. The finding of this large cytoplasmic re-entrant loop prompts the possibility of periplasmic re-entrant loop(s) in this membrane protein. A similar approach can be used on the right-side-out membrane vesicles of cysteine mutants to study the periplasmic loops. Additional methods include split protein constructs with split sites on the putative loops (if the loop is re-entrant, the split protein would likely be inactive) and protease cleavage of the putative loop (cleavage site would likely be hidden from the protease if on the re-entrant loop).

The hydropathy profile of the melibiose carrier [4] showed that the region 360–380 which comprises part of loop X/XI was a small upward bump, located between two major upward peaks presumably representing hydrophobic transmembrane helices X and XI. This is consistent with the hydropathy profile of the postulated re-entrant loop VI/VII of glutamate transporter [37]. Pore-loop structures, like transmembrane helices, also appear in hydropathy profile as peaks, but are usually less hydrophobic [37]. Although there is a remote possibility that the secondary topological model for this particular loop is incorrect, in that part of the X/XI loop is in the transmembrane segment, it is unlikely so, because the cysteine scanning result of the loop fits none of the three types of the known helices previously revealed, namely the helix is either entirely [17] or partially [19,21] in

the aqueous channel, or entirely out of the aqueous channel [20]. The secondary structure of the re-entrant loop in this study, as in other transport proteins, is not clear, though α-helical structure was proposed for part of the extracellular loop of the type II Na^+/Pi cotransporter [35].

A recent study of the cytoplasmic loop IV/V in the MelB from Leblanc lab [47] indicated that functional residues R141 and R149 are involved in sugar translocation and sugar binding, respectively. The study also suggested that loop VI/V undergoes conformational change upon the binding of the substrate. This is consistent with our finding of the conformational change of loop X/XI upon the binding of the substrate, as deduced from the cross-linking pattern of the cysteine mutants on the loop (Figs. 2 and 3B). Since loop VI/V and loop X/XI are near the N- and C-terminus, respectively, the finding that both loops are functionally important in sugar transport may suggest a cooperation between the two halves of the protein in the transport process, and proximity between the functional residues on these two loops in the 3-D structure of the protein.

In contrast to the proton-coupled lactose permease in *E. coli*, which has been described as two structurally symmetrical interacting half proteins with general homology presumably as a result of an ancient gene duplication [43], the sodium-coupled melibiose carrier is structurally asymmetrical as revealed by electron cryomicroscopy [25]: the two halves of the protein have neither much sequence homology nor structural similarity surrounding a curved central cleft (presumably aqueous channel). It is possible that this large cytoplasmic re-entrant loop X/XI contributes to the scaffolding for the asymmetrical aqueous channel.

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